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# Seasonal Relationships between Plasma and Fecal Testosterone in Response to GnRH in Domestic Ganders

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In two groups (eight individuals each) of socially acquainted, outdoor-kept, domestic ganders (male Hungarian white: *Anser domesticus)*, basal and GnRH-stimulated plasma testosterone (T) concentrations were compared with fecal testosterone metabolites (TM) in and between three seasons, spring peak of reproductive activity, summer photorefractoriness, and fall sexual reactivation. Plasma was sampled 90 min following the challenge and T was analyzed by radioimmunoassay following the GnRH challenge. Fecal TM were measured by enzyme immunoassay using two group-specific antibodies against 17$\beta$-OH-androgens or a novel antibody against 17-oxo groups, which was found to react with major testosterone metabolites without prior hydrolytic deconjugation. Baseline plasma T and systemic levels were high in spring and fall but low in summer. Plasma T increases in response to GnRH were followed by significantly elevated fecal TM levels 2 to 6 h following the challenge in spring and fall. In fall, at high plasma T levels, fecal TM levels were disproportionally lower than in spring. Variability of TM levels was two to five times higher in feces than in plasma, which explains why correlations between individual plasma T and fecal TM levels generally remained nonsignificant. This points to a low-level short-term relationship between the excreted TM and the plasma T levels. However, the reliability of the method was demonstrated by standard inter- and intraassay variablilities and by a high correspondence between results obtained by the two assays. It is suggested that, with appropriate sample size, fecal TM reflects plasma T increase. However, fecal TM was more variable than the plasma T, and fecal TM responses to GnRH did not always parallel the plasma T response. In addition, seasonal changes in androgen excretion regimes must be taken into account. 

*Key Words: Anser domesticus;* behavioral endocrinology; EIA; feces; GnRH challenge; noninvasive endocrine monitoring; 17$\beta$-OH-androgen; reproductive cycle; seasonality; social challenge; waterfowl.

Determining hormones from blood samples provides accurate snapshots of plasma concentrations but has obvious drawbacks. Animals must be caught and handled, which may be unfeasible or even dangerous. Handling causes stress and therefore may interfere with the measurements, particularly if repeated sampling is required (Miller *et al.,* 1991). These problems may be overcome by determining steroid hormones or their metabolites from sputum, feces, or urine. Mainly because of these benefits and due to methodological progress (e.g., Möstl *et al.,* 1987; Palme and Möstl, 1993), the noninvasive approach has gained increasing importance in recent years (Wasser *et al.,* 1993; Whitten *et al.,* 1998). For example, this methodology significantly eases field studies (Bujalska *et al.,* 1994; Creel *et al.,* 1996; Hirschenhauser *et al.,* 1999a,b, 2000; Kirk-



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patrick *et al.*, 1992; Kotrschal *et al.*, 1998, Wallner *et al.*, 1999; Wasser *et al.*, 1997; White *et al.*, 1995).

In mammals and birds, the determination of gonadal steroid and corticosteroid metabolites in feces allows gender determination (Bishop and Hall, 1991; Cockrem and Rounce, 1994; Hurst *et al.*, 1957), facilitates monitoring of reproductive status in zoo animals and domestic livestock (Bercovitz *et al.*, 1982; Hultén *et al.*, 1995; Kirkpatrick *et al.*, 1990; Lucas *et al.*, 1991; Möstl *et al.*, 1984; Palme *et al.*, 1996; Schwarzenberger *et al.*, 1992; Ziegler *et al.*, 1988), and is a valuable tool for monitoring stress (Altmann *et al.*, 1995; Bujalska *et al.*, 1994; Cook *et al.*, 1996; Creel *et al.*, 1997; Goymann *et al.*, 1999; Kotrschal *et al.*, 1998; Miller *et al.*, 1991, Wasser *et al.*, 1997). Excreted steroid metabolites may covary with social factors (Hirschenhauser *et al.*, 1999a,b, 2000; Kotrschal *et al.*, 1998; McLeod *et al.*, 1991; Oliveira *et al.*, 1996; Wingfield *et al.*, 1997).

However, the validity of most of this work relies on the time course of excretion (Mac Donald *et al.*, 1983; Peter *et al.*, 1996; Prop and Vulnik, 1992) and on the assumption that the steroid metabolite concentrations from urine or feces proportionally reflect plasma levels. This assumption seems reasonable as long as the noninvasive method yields biologically relevant results. For example, recent investigations on seasonal patterns of steroid metabolites (estrogen, testosterone, corticosterone, progesterone) in greylag geese (*Anser anser*) revealed specific relationships between gender and social status (Hirschenhauser *et al.*, 1997, 1999a, 2000; Kotrschal *et al.*, 1998).

In a limited number of studies, it has been shown that hormone metabolites excreted via feces or urine covary with plasma hormone titers (Bishop and Hall, 1991; Cockrem and Rounce, 1994; Hultén *et al.*, 1995; Miller *et al.*, 1991; Palme *et al.*, 1996; Wasser *et al.*, 1993, 1997; White *et al.*, 1995). The present results from domestic ganders are in line with these findings. However, the time course of excretion varied widely between species and was faster via urine than via feces, in which it depended on gut passage times and other factors. Steroid metabolites reach the gut via liver and bile (Helton and Holmes, 1973; MacDonald *et al.*, 1983; Tailor, 1971). Peak excretion was in the range of days in mammals (Goymann *et al.*, 1999; Hultén *et al.*, 1995; Miller *et al.*, 1991; Palme *et al.*, 1996; Wasser *et al.*, 1993) and in the range of hours in birds (Bishop and Hall, 1991; Cockrem and Rounce, 1994; Kikuchi *et al.*, 1994; Wasser *et al.*, 1997).

In studies of the relationships between steroid hormones and social behavior in free-ranging greylag geese, it became necessary to evaluate the levels of excreted testosterone metabolites (TM) relative to plasma testosterone levels. In a series of experiments and at three times of the year, the reproductive season in spring, the photorefractory period in summer (Peczely *et al.*, 1993), and the peak of sexual activity in fall (Do thi Dong Xuan *et al.*, 1995; Peczely *et al.*, 1994), the quantitative relationships between the plasma testosterone levels and the fecal TM levels were examined. The responses to GnRH stimulation were compared with preexperimental baseline levels in groups of domestic ganders.

## METHODS

### *Animals and Sampling*

Experiments were performed with two outdoor-kept groups of male domestic geese (Hungarian white, *Anser domesticus*) at three different times of the year, end of March (spring peak in reproductive activity), end of July (summer photorefractoriness and postnuptial molting, Péczely *et al.*, 1993), and middle of November (fall sexual reactivation; Dittami, 1981; Hirschenhauser *et al.*, 2000; Peczely *et al.*, 1994). A third, unmanipulated group of ganders was available as a control only in the fall.

Ganders were housed at the Gödöllö Agricultural University in groups of 8 in approximately 40-$m^2$ outdoor pens that had concrete floors and were fenced with wire mesh. Birds had *ad libitum* access to water and food. Groups were put into their pens 1 month ahead of the first experiment in March and were kept within the same groups for the rest of the year. Therefore, animals were well habituated to their situation and to the presence of humans. Ganders were individually marked with colored plastic leg and neck rings and aluminium neck rings with identification numbers.

During each of the three experimental periods, two groups were injected in the pectoral muscle with 10 $\mu$g superactive GnRH analogue (Ovurelin; Reanal, Hun-

gary) per individual in the morning (08:00 to 11:15, depending on group and season). Blood was sampled from the wing veins of these ganders into heparinized polyethylene tubes just before the time of injection (baseline value) and 90 min thereafter, which corresponds with maximal plasma stimulation (Hargitai *et al.,* 1993; Péczely, 1989). Blood samples were drawn within 30 s after capture and immediately centrifuged and the plasma was stored at −20°C.

To collect individual feces as completely as possible throughout the experimental days, the floors of the pens were covered with plastic sheeting and each pen was continuously monitored from the outside by two observers from 7:30 a.m. to 5:00 p.m. Times varied slightly according to season. Over 90% of the droppings could be retrieved by the collectors. Samples were frozen at −20°C in small plastic bags within 1 h of collection.

### *Plasma Hormone Analysis*

Plasma testosterone was analyzed by RIA after extraction with diethyl ether (Péczely, 1989). Values are given in pg/ml plasma. The intra- and interassay coefficients of variation were 11 and 14%, respectively ($n = 52$).

### *Fecal Analysis*

In contrast to chickens (*Gallus gallus;* Kikuchi *et al.,* 1994; Wallpach, 1998), HPLC separation revealed that virtually all of the androgen metabolites in the feces of geese were conjugated (Krawany, 1996). Thus, samples had to be deconjugated (Hirschenhauser *et al.,* 1999a) because the antibody against 17$\beta$-OH-androgens (see below) would not bind to the conjugated metabolite. Deconjugation was done by adding 500 $\mu$l of a 1:500 diluted mixture of $\beta$-glucuronidase/arylsulfatase (Merck 4114) to the methanol-extracted samples. This led to 36.0 ± 14.5% deconjugation of the androgen, however, the enzyme may also have converted part of the steroid itself (Makin *et al.,* 1995).

Fecal testosterone metabolites were then determined by enzyme immunoassay (Möstl *et al.,* 1987) with a group-specific antibody against 4-androstene-17$\beta$-ol-3-on-carboxymethyloxine-albumine-CMO (rabbit). As label, 5$\alpha$-androstane-3$\beta$ (Palme and Möstl, 1993) was used for biotinylation (dioxaoctane biotin). Assay concentration limits for reliable measurements ranged from 0.4 to 30 pg/well. The specificity of this androgen assay was described elsewhere (Hirschenhauser *et al.,* 1999b). The mean intra- and interassay coefficients of variation for the antibody against 17$\beta$-OH androgens were 8.8 and 11.2%, respectively ($n = 328$).

As a test of the results obtained by the antibody, a selection of samples (three individuals per season chosen on the basis of their responses to GnRH and the availability of sufficient fecal samples) was reanalyzed with a recently developed group-specific epiandrosterone antibody against 17-oxo groups, which includes the 17$\beta$-OH-androgens (E. Möstl, unpublished). This antibody allows a direct assay (without the necessity of a deconjugation step). It significantly increased the amount of metabolites recognized compared to that of the "old" antibody and thus also increased the sensitivity of the assay. Still, the results obtained with the new antibody varied in parallel with the results obtained by the old antibody and therefore validated the results obtained by the latter. The intraassay coefficient of variation for the 17-oxo antibody ($n = 45$) was 10.9% as determined by homogenized pooled samples.

### *Separation of Hormone Metabolites by HPLC*

To analyze the excretory metabolites of testosterone, 370 kBq [$^{14}$C]testosterone (NEC-101, New England Nuclear) dissolved in 6 ml sterile 0.9% NaCl solution containing 10% (v/v) ethanol was injected i.v. into each of two ganders. All droppings were collected from the plastic sheeting of the floor of the enclosure. From each of the droppings, 0.5 g was extracted with 5 ml 80% methanol and centrifuged, and the radioactivity of the supernatant was measured by liquid scintillation counting.

One milliliter of the supernatants of the samples with the highest radioactivity was diluted with 10 ml water and prepurified using SepPac C18 cartridges according to the recommendations of the manufacturer. Steroids were eluted from the column with 4 ml methanol. After evaporation, reversed-phase chromatography was performed (column, Novopac C18, 3.9 × 150 cm; eluent, 20% methanol for 5 min, then linear increase to 100% methanol in 30 min; flow rate 1 ml/min, 3 fractions/min). Half of the volume of

each fraction was transfered into a new vial and the mobile phase of the chromatograms was evaporated. In one half of the chromatogram, the enzymatic hydrolysis was performed overnight. Immunoreactive substances were measured in all fractions (with and without hydrolysis) using enzyme immunoassays for 17$\beta$-OH-androgens and 17-oxo-androgens (Palme and Möstl, 1993).

The radioactivity eluted from the column in three peaks with maxima in the 30th, 39th, and 46th fractions. Using the 17$\beta$-OH-androgen assay, immunoreactive substances were measured predominately after hydrolysis. The immunoreactive peaks coeluted from the column together with the radioactive material in the first 2 fractions. Using the assay for the 17-oxo-androgens, the 3rd fraction was immunoreactive. Enzymatic hydrolysis overall diminished the immunoreactivity.

The HPLC immunogram indicates that all three main radioactive peaks show immunoreactivity. Therefore, both assays react with testosterone metabolites excreted in the feces. The dominating radioactive peak was detected by the 17-oxo assay without prior hydrolysis.

### *Gut Passage Time, Sampling Scheme, and Data Processing*

The lag between a plasma hormone peak and its appearance in the feces depends on gut passage time. Charcoal dust-marked food revealed a gut passage time of 2–5 h (unpublished), which is in line with data from the literature (Mattocks, 1971; Prop and Vulink, 1992). Indeed, individual fecal peak values appeared during this time period (Figs. 1b and 2). Therefore, the individual mean testosterone metabolites of samples collected within 2 h of the challenge were considered as intraindividual "baseline" controls. Baseline plasma and fecal TM levels were compared with response levels and both were compared between seasons either by ANOVA with Bonferroni post hoc testing or by Wilcoxon test for within-category comparisons (Fig. 5). In addition to mean fecal TM responses, baseline values were compared with individual maxima (peaks) values between 2 and 6 h after GnRH injection (Figs. 1 and 5).

The use of excreted steroid hormone metabolites, particularly for investigating short-term interactions between behavior and hormones, relies on the assumption that the temporal variation in hormone metabolite levels obtained from fecal samples reflects the variation in hormone levels found in plasma. Therefore, plasma T values were correlated with fecal TM values by Spearman's rank correlation.

Sample sizes vary (e.g., Fig. 5) due to variable defecation rates during the baseline interval; only complete data pairs (for a given individual, samples were available for both periods) were considered.

## RESULTS

### *Defecation Rates*

Defecation rates of individuals over a sampling day of approximately 10 h were similar in spring and summer ($4.65 \pm 2.84$, $n = 60$ and $3.44 \pm 2.46$, $n = 57$) but were considerably higher in fall ($8.28 \pm 3.99$, $n = 58$); more gander groups were kept and sampled than considered in this paper, hence the high number of individuals.

### *Response Times of Plasma Testosterone and Fecal Testosterone Metabolites to the GhRH Challenge*

Whereas the plasma peaks were sampled 90 min after the GnRH injection (Péczely, 1989), peak values appeared in the feces 3–6 h following the challenge (Fig. 1). Maximum fecal testosterone metabolite excretion varied between individuals and seasons (Fig. 2). Time lags between the stimulation and the appearance of fecal maximum TM levels were $181 \pm 66$ min in spring, $350 \pm 131$ min in summer, and $218 \pm 58$ min in fall.

### *Seasonal Effects*

With both antibodies, the 17-$\beta$-OH-androgen and the 17-oxo-androgen, responses of plasma TM to GnRH injection were significantly different between seasons (Fig. 3), highest in spring, intermediate in summer, and lowest in the fall, but results varied in parallel. Thus, even though the novel 17-oxo-androgen antibody was considerably more sensitive, it validated the results obtained with the "old" 17-$\beta$-OH-androgen antibody. The 17-oxo assay detected the

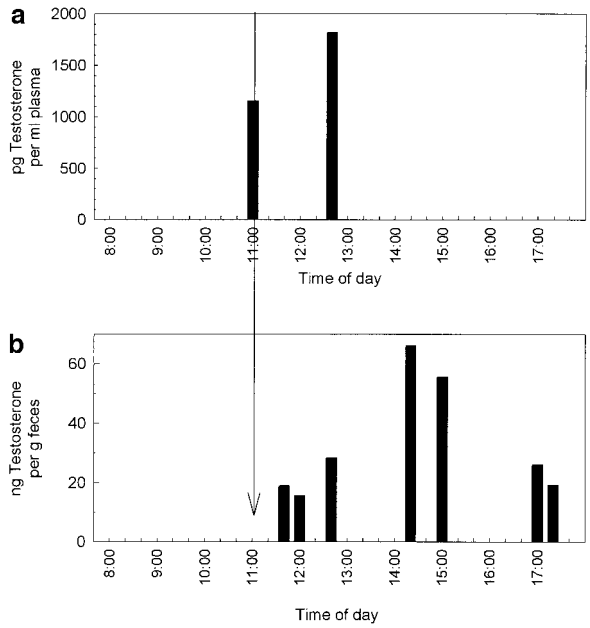


FIG. 1. Example of individual plasma testosterone values (a) by RIA and fecal 17$\beta$-OH androgen values (b) by EIA in response to GnRH application (arrow) over the course of a spring day. Plasma was sampled 90 min after the GnRH challenge, at the time of the plasma T maximum (as found by Peczely, 1989). Individual No. 47 is shown.

major peak (Fig. 4; see Methods). It is concluded that both assays measure fecal testosterone metabolites.

In spring and fall, but not in summer, plasma T was significantly increased compared with baseline values 90

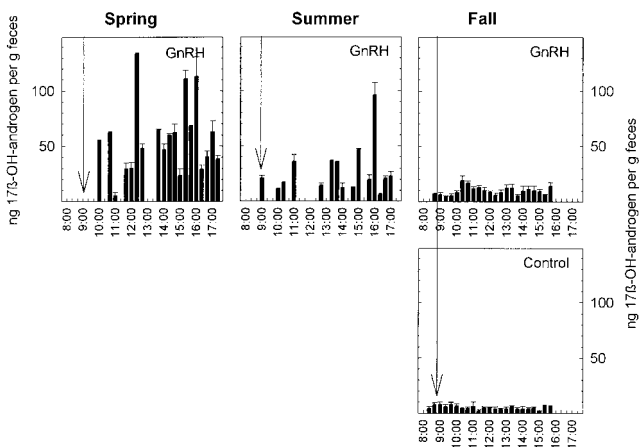


FIG. 2. Group mean ($\pm$SE) fecal 17$\beta$-OH-androgen (TM) of 20-min intervals over the day in spring, summer, and fall. GnRH injection (top) and unhandled control (bottom, available only in fall). Time of injection shown by the arrow. As individual defecation rates are irregular, the number of samples integrated per bar ranged between 1 and 8. In bars lacking *SE*, $n = 1$.

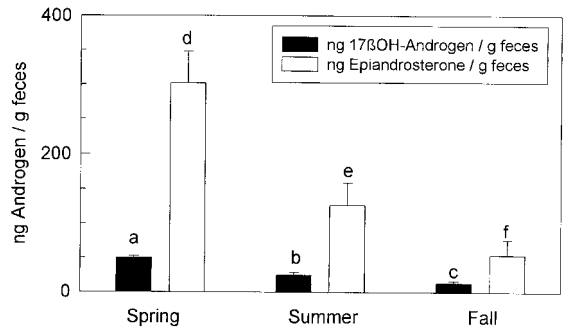


FIG. 3. A seasonal comparison of ng androgen / g feces (means + SE) obtained by parallel analysis of samples by the two different antibodies. Samples were taken from the same three individuals each season chosen on the basis of their distinct responses to GnRH and the availability of sufficient individual fecal samples (compare to Fig. 5). The 17$\beta$-OH antibody necessitated deconjugation prior to analysis and was used throughout this study. The novel epiandrosterone 17-oxo antibody allowed a direct assay and was applied only to a subsample of this study. The epiandrosterone antibody revealed significantly higher values in all seasons (Wilcoxon, two-tailed: $Z = -2.67$, $n = 9$, $P = 0.008$).

min after GnRH injection (Fig. 5a). Also, the baseline plasma T levels were significantly higher in spring (at the peak of sexual activity) and in late fall (sexual reactivation) than in summer (during photorefractoriness) (Fig. 5a).

In all three seasons, fecal TM metabolites tended to be higher following the GnRH challenge than before

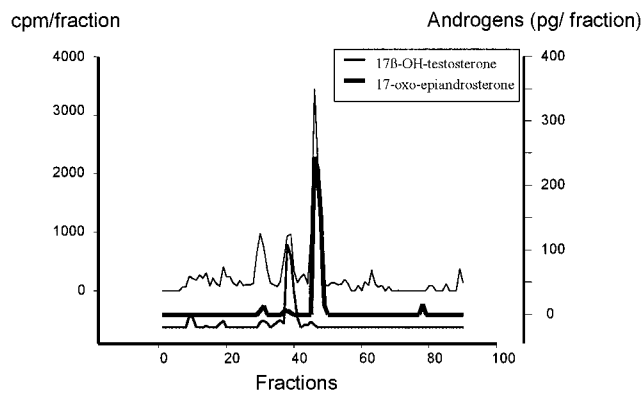


FIG. 4. HPLC immunogram after i.v. application of [$^{14}$C]testosterone into a goose. The fecal sample with the the peak radioactivity 149 min after injection was separated first via a Seppac C18 column, followed by RP-HPLC. The 17$\beta$-OH-androgen assay bound to a few minor metabolites, whereas the 17-oxo-androgen assay detected the major metabolite peak. Both assays reliably measure fecal testosterone metabolites.

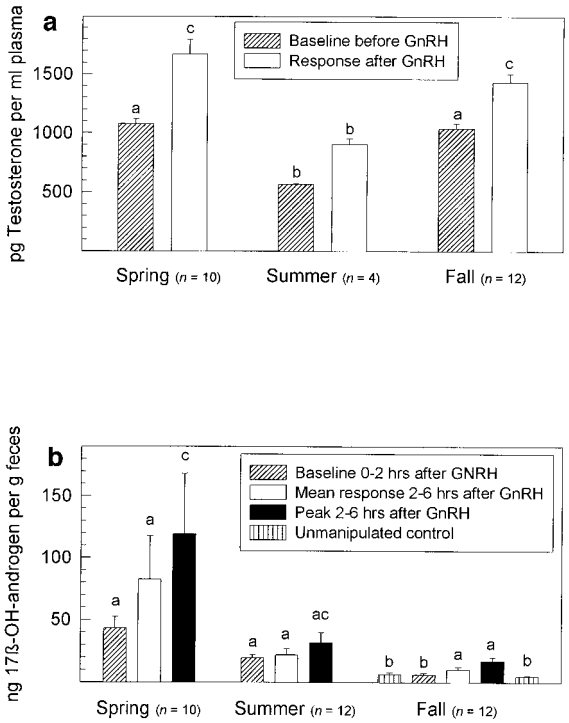


FIG. 5. Seasonal comparison (means + SE) of baseline testosterone before GnRH stimulation and responses measured in plasma (a), sampled 90 min after injection, and in feces (b), baseline sampled 0–2 h after stimulation and mean response from samples within 2–6 h after stimulation. An unmanipulated control group was available for feces sampling only in fall. Two groups of eight ganders were available per season and treatment. Sample sizes vary because individuals were included only when both baseline and response values were available (depending mainly on irregular defecation). Significant differences are indicated by different letters, a–c. In the plasma (a), both baseline and response T differed significantly between seasons (one-way ANOVA: baseline, $F = 24.32$, $df = 2$, $P = 0.000$; response, $F = 9.14$, $df = 2$, $P = 0.0012$); spring and fall levels significantly exceeded summer levels (Bonferroni post hoc test). Within seasons, response T significantly exceeded baseline T in spring and fall (Wilcoxon: spring, $Z = -2.803$, $n = 10$, $P = 0.005$; fall, $Z = -3.0594$, $n = 12$, $P = 0.002$) but nonsignificantly so in summer ($Z = -1.826$, $n = 4$, $P = 0.068$). In the feces (b), baseline TM was significantly different between seasons (one-way ANOVA: $F = 10.455$, $df = 2$, $P = 0.0006$); spring baseline was significantly higher than fall baseline (Bonferroni post hoc test). Seasonal differences of mean TM responses and peaks were only marginally significant ($F = 3.182$, $df = 2$, $P = 0.06$ and $F = 3.125$, $df = 2$, $P = 0.063$). Peak TM levels significantly exceeded baseline levels in spring (Wilcoxon: $Z = -2.395$, $n = 10$, $P = 0.017$). In fall, both mean responses and peaks significantly exceeded baseline TM (mean response, $Z = -2.04$, $n = 12$, $P = 0.041$; peak, $Z = -3.061$, $n = 12$, $P = 0.002$), whereas in summer, even the peaks did not significantly exceed baseline TM ($Z = -0.73$, $n = 4$, $P = 0.465$).

TABLE 1

Spearman's Rank Correlations ($r_s$) between Individual Plasma Values (pg/ml) and Fecal 17β-OH Androgen (ng/g)

| | Spring | Summer | Fall |
|---|---|---|---|
| Baseline | $r_s = 0.12$ | $r_s = -0.4$ | $r_s = 0.03$ |
| | $n = 10$ | $n = 4$ | $n = 12$ |
| | $P = 0.751$ | $P = 0.6$ | $P = 0.931$ |
| Mean response | $r_s = 0.26$ | $r_s = 0.2$ | $r_s = -0.53$ |
| | $n = 10$ | $n = 4$ | $n = 12$ |
| | $P = 0.467$ | $P = 0.8$ | $P = 0.074$ |
| Peaks | $r_s = 0.38$ | $r_s = 0.2$ | $r_s = -0.38$ |
| | $n = 10$ | $n = 4$ | $n = 12$ |
| | $P = 0.275$ | $P = 0.8$ | $P = 0.225$ |
| First fecal sample | $r_s = -0.23$ | $r_s = -0.49$ | $r_s = -0.3$ |
| | $n = 16$ | $n = 10$ | $n = 14$ |
| | $P = 0.387$ | $P = 0.15$ | $P = 0.302$ |

*Note.* Baselines (0–2 h after GnRH), mean responses (2–6 h after GnRH), peaks (within 2–6 h after GnRH), and first fecal sample after the plasma peak (90 min after GnRH). Sample sizes vary between seasons because only complete pairs of values were included in the analysis.

the challenge (Fig. 5b). However, only in the fall did the mean response values significantly exceed baseline. In spring and fall, peaks were significantly higher than baseline values. Despite the significant results obtained for fecal TM in fall, excreted amounts in fall were still disproportionally low compared to the relationship between plasma and fecal TM in spring (Fig. 5). Despite similar baseline plasma T concentrations in spring and fall (Fig. 5a), the mean excreted TM in spring exceeds the mean fecal fall response by approximately 13-fold and the spring excreted TM peak exceeds the fall fecal peak by 8-fold.

### *Variability in Plasma and Feces and Correlations between Plasma and Feces*

Intraindividual variability of TM in response to GnRH challenge was considerably higher in feces than in plasma (Fig. 5). Coefficients of variation were 12% in plasma and 166% in feces in spring, 27 and 125% in summer, and 22 and 58% in fall.

As a measure of short-term association between plasma T and fecal TM levels, fecal TM should be significantly correlated with plasma T over the course of a day. However, based on individual data, no significant correlations were found (Table 1).

A reanalysis of samples from three GnRH-challenged individuals per season using the novel group-

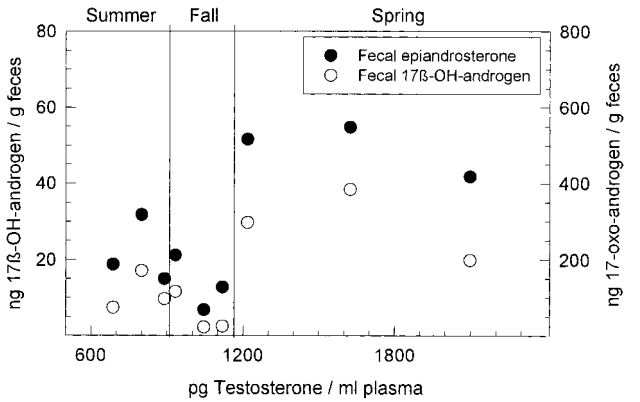


FIG. 6. Scatterplot of the relationships between pg testosterone / ml plasma (x axis) and ng androgen metabolites / g feces as measured by EIA with the 17$\beta$-OH antibody (which necessitated deconjugation prior to analysis and was used throughout this study) and with the new epiandrosterone 17-oxo antibody (which allowed a direct assay and was applied only to a subsample of this study) (compare to Fig. 3). Results of three individuals per season stimulated with GnRH. Correlations between plasma and fecal androgens were not significant (Spearman's rank correlation, $n = 9$: 17$\beta$-OH antibody, $r_s = 0.47$, $P = 0.21$; 17-oxo antibody, $r_s = 0.5$; $P = 0.17$) and remained so even after removal of the fall values (Spearman's rank correlation, $n = 6$: 17$\beta$-OH antibody, $r_s = 0.66$, $P = 0.156$; 17-oxo antibody, $r_s = 0.77$; $P = 0.072$). The fecal metabolite values measured by the two different antibodies with the same samples are highly correlated (Spearman's, $r_s = 0.98$, $P < 0.000$, $n = 9$).

specific epiandrosterone antibody yielded parallel patterns (Fig. 6). The results for the same individuals analyzed using both antibodies were highly correlated (Spearman's $r_s = 0.98$, $n = 9$, $P < 0.000$), which underlines the validity of the analysis using the old antibody. However, when correlated with plasma values, Spearman's $r_s$ (at $n = 9$) was 0.47 for the 17-$\beta$-OH-androgen and 0.5 for the 17-oxo-androgen; both correlations remained nonsignificant (see Fig. 6), which may have been due to the low fecal TM measured in fall. Excluding these fall values, the correlations of both the old and the new antibodies with the plasma values approached significance levels (Fig. 6).

## DISCUSSION

Stimulation with GnRH produced a consistent, time-lagged response of excreted androgen metabolites following prior plasma T peaks. Thus, GnRH treatment had the expected effects on fecal testosterone metabolites. However, there were also unexpected effects of season. Amounts of excreted TM in relation to the plasma T concentrations were disproportionally low in fall compared to spring. Still, the significant effects of GnRH stimulation on plasma T in spring and fall translated into significantly increased peak (spring and fall) and mean response (fall) levels compared to baseline TM levels in feces (Fig. 5). However, the considerable variability of fecal TM may have precluded significant short-term correlations between plasma T and excreted T metabolite levels.

### *Seasonality*

Seasonality of reproduction in geese (Hirschenhauser *et al.,* 1999b, 2000) probably causes high plasma T responsiveness to GnRH stimulation in spring (Figs. 1 and 2; Dittami, 1981; Hargitai *et al.,* 1993) but also in fall, when the hormonal system resumes sexual and social responsiveness in preparation for the following spring reproductive period (Hirschenhauser *et al.,* 1999a; Peczely *et al.,* 1993; Wingfield and Farner, 1980). The fecal TM response was in proportion to the plasma T response in spring and summer but was much lower in fall (Fig. 5). Similar seasonal effects of environmental / social stimuli were also recorded in pied flycatchers (*Ficedula hypoleuca;* Silverin, 1998), in which particularly the unpaired males showed high T levels (Hirschenhauser *et al.,* 1997, 1999a; Kotrschal *et al.,* 1998; Oliveira *et al.,* 1996; Wingfield *et al.,* 1990).

Since the same seasonal pattern of excretion was found with both antibodies used, it is assumed that the low fall TM excretion is not an artifact of the assay procedure. Low fall TM concentrations in feces may simply be related to the higher defecation rates in fall than in spring or summer (see Results). If some constant amount of TM is excreted per unit time, a near doubling of the defecation rate and therefore of fecal volume, as is indeed the case in fall, would accordingly dilute fecal TM concentration. However, fall fecal TM concentrations dropped to less than 20% of the spring values, which cannot be due to increased fecal volume alone.

Probably, this seasonal pattern is not an effect of a general metabolic down-regulation or of a specifically low T production in fall, because plasma T in response to GnRH was approximately as high as that in spring. However, the possibility remains that in fall both pro-

duction and clearance of T from the system are decreased, which would explain the discrepancy between the relatively high levels of plasma T and the low levels of excreted TM. Still, fall feces peaks appeared with time lags after the challenge similar to those in spring, and defecation rates, probably due to seasonally increased food intake associated with fattening, were even higher in fall than in the other seasons (Prop and Vulink, 1992).

There are still other potential mechanisms explaining the relatively low TM excretion rates in fall. For example, higher uptake of T by target organs in fall compared to spring (mallard drakes, *Anas platyrhynchos;* Horst and Paulke, 1977) may have buffered GnRH-related T surges before excretion. In addition, the levels of circulating binding proteins and therefore the capacity to buffer T surges in the plasma may vary with season (Silverin, 1998).

A plausible functional explanation for the difference in TM excretion rates between spring and fall may be that spring is the active sexual season and high clearance rates are required to keep the animals behaviorally responsive. In fall, stimulation (both physiological and social) may cause high plasma levels comparable to those in spring. However excretion may be slower in fall than in spring because it may be less important in fall to optimize the animals' short-term behavioral responsiveness compared to that in the spring reproductive period.

Whatever the physiological causes and functional consequences of this seasonally different excretion regime may be, caution regarding the interpretation of seasonal comparisons of excreted TM is justified. For example, on the basis of the present results, it may be assumed that the fall peak in seasonal fecal androgen metabolites in Greylag geese as described by Hirschenhauser *et al.* (1999a) may underrepresent the amounts of circulating levels, because plasma T levels in fall were not different from those in spring (Fig. 5a) and yet fecal TM levels were lowest in fall (Fig. 5b). This does not apply to spring, in which the fecal peak reflects the plasma peak.

### *Sources of Variability of the Fecal Androgen Metabolites*

The variability in excreted TM and thus the low-level short-term correlation between plasma T titers and fecal T metabolite levels may have a number of reasons, such as the assay procedure itself, the steroid hormone metabolism, the excretion dynamics via the gut, and the fact that the urine cannot be completely separated from the fecal component of a goose dropping. Urine and feces may have different excretion regimes and the transfer of metabolites into the gut via bile necessarily increases variability.

The assay procedure itself was probably not the major component of high fecal TM level variability but certainly contributed to it. Every step of the procedure, from weighing the feces (which may differ in their water content) to spectrophotometry, adds variation. This also applies to the deconjugation procedure. As in cockatiels (*Nymphicus hollandicus;* Tell, 1997), but in contrast to domestic fowl (Cockrem and Rounce, 1994; Wallpach, 1998), virtually all excreted steroids were found to be conjugated in geese (Krawany, 1996). Still, the reliability of our assays is underlined by its standard intra- and interassay variabilities (see Methods), as well as by the strong correlation between the results obtained with the 17$\beta$-OH antibody, which necessitated deconjugation, and the results obtained with the novel 17-oxo antibody, which did not.

The metabolism in the liver (MacDonald *et al.*, 1983; Peter *et al.*, 1996; Taylor, 1971) and renal or intestinal excretion (Helton and Holmes, 1973; Krawany, 1996) may explain why even the significant covariations between the plasma hormones and their excreted metabolites reported in the literature are based on moderate correlation coefficients between 0.4 and 0.8 (Bishop and Hall, 1991; Cockrem and Rounce, 1994; Hultén *et al.*, 1995; Miller *et al.*, 1991; Wasser *et al.*, 1993). In baboons (*Papio cynocephalus;* Wasser *et al.*, 1993) as well as in pigs (Hultén *et al.*, 1995) and sheep (Palme *et al.*, 1996), the time lag between the appearance of progesterone in blood and its peak appearance in feces was 1–2 days. Significant interspecies differences were found in the time course and pathway of excretion, via urine or feces (Kikuchi *et al.*, 1994; Peter *et al.*, 1996; Whitten *et al.*, 1998).

With respect to excretion, other steroid hormones, such as corticosterone (B), may be comparable to T. Peak urine excretion of infused [$^{14}$C]cortisol, for example, occurred after approximately 12 h in sheep (*Ovis ammon domesticus*), after 24 h in ponies (*Equus caballus*), and only after 48 h in pigs (*Sus scrofa domestica;* Palme

*et al.,* 1996). In bighorn sheep (*Ovis canadensis*), the urinary cortisol peak appeared 2–6 h after an ACTH challenge (Miller *et al.,* 1991). Fecal glucocorticoid responses were observed 24–50 h following an ACTH challenge in spotted hyenas (*Crocuta crocuta;* Goymann *et al.,* 1999). In an owl, injected ACTH caused a fecal B peak after 12 h (*Strix occidentalis caurina;* Wasser *et al.,* 1997). In domestic geese, however, such a challenge produced fecal responses only after 4–5 h (Kotrschal *et al.,* unpublished). The reason for this difference between two avian species is probably the shorter gut passage time in the herbivorous geese.

After injection of 3H corticosterone, marked metabolites appeared in the droppings of domestic and greylag geese after just 10 min and peaked within 2 h (Krawany, 1996; Möstl, unpublished). This fast excretion may have been via urine. From visual inspection, the white component of the dropping was restricted to the outside and certainly did not exceed the 8% reported in droppings from barnacle geese (*Branta leucopsis;* Prop and Vulink, 1992). Still, the urine contents of the droppings sampled may have varied. The present measurements therefore always integrate both excretory pathways, urinary and fecal. This seems acceptable, since in geese there is no long time lag between the (fast) urinary excretion and the (slow) fecal excretion caused by the gut passage times of most mammals.

Due to the short gut passage time of 2–5 h in domestic geese (Mattocks, 1971; Prop and Vulink, 1992), the temporal resolution of the method is potentially good (but see correlations in Table 1). Seasonal variability in gut passage times in geese may be caused by different feeding regimes. Particularly in fall, food uptake is high (Prop and Vulink, 1992) and, consequently, gut passage time is relatively short. In contrast, food uptake is relatively low during molt in summer and, consequently, gut passage time is long. However, seasonally varying feeding regimes and fecal volumes may lead to changes in fecal TM concentrations.

In summary, fecal TM levels were more variable than plasma T levels. Concomitantly, the present study failed to demonstrate close correlations between plasma T and fecal TM levels. Therefore, it is suggested that sufficiently high sample sizes are critical when short-term relationships between hormones and behavior are examined. Still, the parallel responses of plasma T and fecal TM to GnRH challenges indicate the general validity of the noninvasive approach. However, caution is appropriate when comparing different seasons or reproductive states.

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